総　説

ヒトにおける尿中 5- アミノレブリン酸とニコチンアミド異化代謝産物排泄量との関係

毛利田　展　也[2]・柴　田　克　己[1]

Relationship between Urinary Excretion Amounts of 5-Aminolevulinic Acid and Urinary Nicotinamide Catabolites in Humans

MORITA Nobuya and SHIBATA Katsumi

Summary： 5-Aminolevulinic acid（5-ALA）is used as a chemotherapeutic agent, and 5-ALA is a direct precursor of heme. Some B-group vitamins are required for the synthesis of 5-ALA from amino acids. 5-ALA is produced by the enzyme 5-ALA synthase, a vitamin B_6-dependent enzyme in mitochondria, from glycine and succinyl-CoA. Succinyl-CoA is mainly synthesized from 2-oxoglutaric acid + CoA by the enzyme 2-oxoglutaric acid dehydrogenase, which needs B-group vitamins such as thiamine diphosphate, FAD, and NAD^+ as coenzymes. Succinyl-CoA is also synthesized from branched chain amino acids and so on. Suitable B-group vitamin administration might increase production of 5-ALA. Here, we investigated the relationships between vitamin nutritional status and 5-ALA production in humans. 5-ALA did not have a significant relationship with the intake of vitamins B_1, B_2, B_6, B_{12} and niacin. Furthermore, urinary excretion of 5-ALA did not correlate with urinary concentrations of vitamins B_1 and B_2. On the other hand, a strong, directly proportional relationship was observed between urinary excretion amounts of 5-ALA and sum of the nicotinamide catabolites such as N^1-methylnicotinamide, N^1-methyl-2-pyridone-5-carboxamide, and N^1-methyl-4-pyridone-3-carboxamide（$r^2 = 0.494$, $P < 0.0001$）. A significant correlation was also found between levels of urinary 5-ALA and 4-pyridoxic acid（a catabolite of vitamin B_6）（$r^2 = 0.220$, $P = 0.012$）. Therefore, administration of niacin and vitamin B_6 might help to increase 5-ALA production.

Key Words： 5-aminolevulic acid, urine, human, vitamin, amino acid

要旨：5- アミノレブリン酸（5-ALA）は，化学療法剤として使用されている．5-ALA はヘムの直接前駆体である．いくつかの B 群ビタミンは，5-ALA 合成に関与している．5-ALA は，ミトコンドリアに存在するビタミン B_6 酵素である 5-ALA 合成酵素によってグリシンとスクシニル -CoA から合成される．スクシニル -CoA は，補酵素としてチアミン二リン酸，FAD および NAD^+ を必要とする 2- オキソグルタル酸デヒドロゲナーゼによって 2- オキソグルタル酸と CoA から主に合成される．さらに，スクシニル -CoA は分岐鎖アミノ酸などからも合成される．適切な B 群ビタミンの補充は，5-ALA の産生を増加させる可能性がある．そこで我々は，ビタミン栄養状態と 5-ALA 産生との関係を調べた．今回のヒトを対象とした調査実験では，尿中 5-ALA は，ビタミン B_1，B_2，B_6，B_{12} およびナイアシンの摂取

[1] 甲南女子大学医療栄養学部医療栄養学科
[2] 滋賀県立大学大学院人間文化学研究科栄養部門修士学生

量とは有意な関係を認めることはできなかった．さらに，尿中の 5-ALA 排泄量は，ビタミン B_1 および B_2 の尿中排泄量と相関しなかった．一方，尿中 5-ALA 排泄量とニコチンアミド異化代謝産物物である N^1- メチルニコチンアミド，N^1- メチル -2- ピリドン -5- カルボキサミドおよび N^1- メチル -4- ピリドン -3- カルボキサミドの合計量排泄量との間には強い正比例関係が認められた（$r^2 = 0.494$, $P < 0.0001$）．さらに，尿中 5-ALA と 4- ピリドキシン酸（ビタミン B_6 の異化代謝産物）量との間にも有意な相関が見られた（$r^2 = 0.220$, $P = 0.012$）．したがって，ナイアシンやビタミン B_6 補充は，ヘムの不足に起因するいくつかの疾患の予防または遅延を助ける可能性がある．

キーワード：5- アミノレブリン酸，尿，人，ビタミン，アミノ酸

Introduction

Increased formation of 5-aminolevulinic acid（5-ALA）in the body might help to prevent or delay some diseases; 5-ALA plus blue light illumination is used for the treatment of various premalignant and malignant lesions with some encouraging clinical outcomes（*1,2*）. The use of 5-ALA to improve glucose intolerance has been considered（*3,4*）. In addition, administration of 5-ALA to mice with sarcopenia improved physical performance（*5*）.

5-ALA is a key intermediate in heme synthesis, and is the first compound in the porphyrin synthesis pathway（Fig. 1）. 5-ALA is produced by the enzyme 5-ALA synthase, a vitamin B_6-dependent enzyme in mitochondria,（*6*）from glycine and succinyl-CoA. Succinyl-CoA is mainly synthesized from 2-oxoglutaric acid + CoA by the enzyme 2-oxoglutaric acid dehydrogenase, which needs B-group vitamins such as thiamine diphosphate, FAD, and NAD^+ as coenzymes. There are two pathways for the production of 2-oxoglutaric acid; the first is from isocitric acid from the TCA cycle, the second is from glutamic acid, glutamine, and some other amino acids（Fig. 1）. In addition, there is another origin of succinyl-CoA, from methylmalonyl-CoA via propionyl-CoA supplied through isoleucine, valine, and methionine（Fig. 1）.

Suitable B-group vitamin intake might increase 5-ALA production. So, we investigated the relationships between nutrient intake and 5-ALA production in humans. We report here that urinary excretion amounts of nicotinamide catabolites such as N^1-methylnicotinamide（MNA）, N^1-methyl-2-pyridone-5-carboxamide（2-Py）, and N^1-methyl-4-pyridone-3-carboxamide（4-Py）were very highly correlated with urinary excretion of 5-ALA. Better niacin nutrition might make production of 5-ALA increase.

Materials and Methods

Chemicals

Thiamine hydrochloride（$C_{12}H_{17}ClN_4OS$-HCl, molecular weight［MW］= 337.27）, riboflavin（$C_{17}H_{20}N_4O_6$, MW = 376.37）, cyanocobalamin（$C_{63}H_{88}CoN_{14}O_{14}P$, MW = 1355.40）, and nicotinamide（$C_6H_6N_2O$, MW = 122.13）were purchased from Wako Pure Chemical Industries（Osaka Japan）, as was 4-pyridoxic acid（4-PIC; $C_8H_9NO_4$, MW = 183.16）manufactured by ICN Pharmaceuticals（Costa Mesa, CA, USA）. 5-ALA hydrochloride（$C_5H_5NO_3$-HCl, MW = 167.6）was purchased from Sigma（St. Louis, MO, USA）, and MNA chloride（$C_7H_9N_2O$-HCl, MW = 159.61）was from Tokyo Chemical Industry（Tokyo, Japan）. 2-Py（$C_7H_8N_2O_2$, MW = 152.15）（*7*）and 4-Py（$C_7H_8N_2O_2$, MW = 152.15）（*8*）were synthesized as described. All other chemicals were of the highest purity available from commercial sources.

Study design

The protocol conformed to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Shiga Prefecture（Shiga, Japan）（approval number 343）. All participants provided written informed consent. Female Japanese students aged 20–21 years were recruited. Average body height, body weight, and

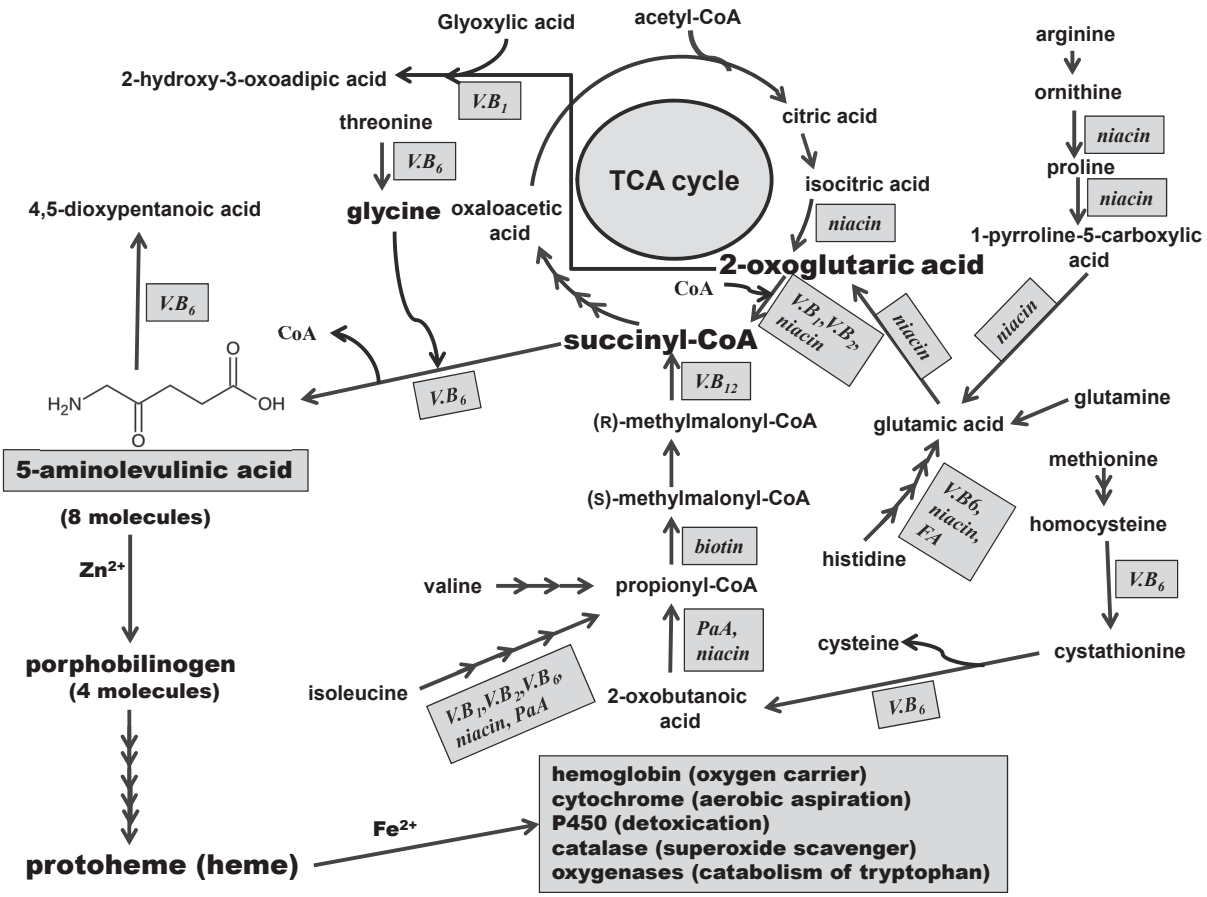

Figure 1. The biosynthesis of 5-aminolevulinic acid（5-ALA）, a precursor of heme, from succinyl-CoA and glycine. 5-ALA biosynthesis requires some B-group vitamins such as vitamin B_1（V.B_1）, vitamin B_2（V.B_2）, vitamin B_6（V.B_6）, vitamin B_{12}（V.B_{12}）, niacin, and pantothenic acid.

body mass index were 156.8 ± 5.0（mean ± SD, n = 28）cm, 49.8 ± 5.1 kg, and 20.3 ± 2.3 kg/m^2, respectively. Individuals with a cold or influenza, or who had taken multivitamin supplements at least once during the previous month, were excluded. All 28 participants were non-smokers, passed a standard medical examination, and completed the study.

The experimental period was 4-days. The experiment was done from Monday to Thursday. All food consumed during the 4-day period was recorded using a weighed food record method（*9*）. All the participants lived and consumed food freely during the experiment. A digital cooking scale（1 g unit; Tanita Inc. Tokyo, Japan）, a set of dietary record forms and a dietary record manual were distributed to the participants in advance（*9*）. Urine samples from the second urination on Thursday to the first urination on Friday were collected in amber bottles by the participants. All the urine samples from an individual were pooled before analysis and used to measure urine B-group vitamins and 5-ALA. After the urine samples were collected, the volumes of the samples were measured on Friday morning in our laboratory. Aliquots of the urine were stabilized to avoid destruction of B-group vitamins and 5-ALA. For analyses of urine thiamine, riboflavin, 4-PIC, nicotinamide, MNA, 2-Py, 4-Py, and 5-ALA, 1 mL of 1 mol/L HCl was added to 9 mL urine. The treated urine samples were then stored at –80℃ until analysis.

Measurement of B-group vitamins in urine

Thiamine. Acidified frozen urine samples（about 600 μL）were thawed and centrifuged at 10,000×*g* for 10 min at 4℃. To each 500 μL supernatant sample was added 1% cyanogen bromide（100 μL）plus 5%（w/v）NaOH（100 μL）. After incubation at room temperature for 10 min, 1.5 mol/L HCl（80 μL）was added, and the mixture was centrifuged at 10,000×*g* for 10 min at 4℃. The resulting supernatant was passed through a 0.45-μm Hydrophilic Durapore™ filter（PVDF; Millipore, Bedford, MA, USA）. A 20 μL aliquot of

filtrate was injected directly into a high-performance liquid chromatography（HPLC）system to measure thiochrome concentration（*10,11*）.

Riboflavin and 4-PIC. Acidified frozen urine samples（about 100 μL）were thawed and centrifuged at 10,000 × *g* for 10 min at 4℃. The supernatants were decanted and passed through a 0.45-μm microfilter. Aliquots of filtrate（20 μL）were injected directly into a HPLC system to measure concentrations of riboflavin（*12*）and 4-PIC（*13*）.

Nicotinamide and its catabolites. Acidified frozen urine samples（2.0 mL）were thawed and centrifuged at 10,000 × *g* for 10 min at 4℃. Each supernatant（1.0 mL）was added to 10 μL of 1.0 g/L isonicotinamide（used as an internal standard）and diethyl ether（5.0 mL）, in the presence of K_2CO_3（1.2 g）, to extract nicotinamide, 2-Py, and 4-Py from the water layer into the organic phase. This extraction procedure was repeated twice. The combined diethyl ether layer was dried at 40℃, and the dried materials were dissolved in 0.5 mL of water. The solution was passed through a 0.45-μm microfilter. Aliquots of filtrate（20 μL）were injected directly into a HPLC system to simultaneously measure nicotinamide, 2-Py, and 4-Py, as described（*8*）.

To measure MNA, urine samples（800 μL）were incubated with 0.1 mol/L acetophenone in ethanol（500 μL）and strong alkali（1.0 mL of 6 mol/L NaOH）in an ice-water bath. The reaction was performed in the presence of a large amount of isonicotinamide（200 μL of 1 mol/L）, which has been reported to interfere with the deamination of MNA under strongly alkaline conditions. Thus, after 10 min, 500 μL of formic acid was added and the mixture was kept for another 15 min in the ice-water bath. The mixture was then immersed in a boiling water bath for 5 min to yield 1-methyl-7-phenyl-1,5-dihydro-5-oxo-1,6-naphthyridine. After cooling in an ice-water bath, the mixture was passed through a 0.45-μm microfilter, and the filtrate（20 μL）injected directly into a HPLC system（*14*）.

Measurement of 5-ALA in urine

An acetylacetone reagent was prepared by mixing 3 mL acetylacetone, 2 mL ethanol, and 15 mL 4 g/L NaCl. Formaldehyde was diluted 3.7-fold in water to 10% and stored in the dark.

Frozen acidified urine samples（100 μL）were thawed and centrifuged at 10,000 × *g* for 10 min at 4℃. The supernatant was withdrawn, and fluorescence derivatization of 5-ALA performed essentially as described（*15*）. To each test tube with a sealed cap was added 20 μL urine supernatant, 5.0 mL acetylacetone reagent, and 0.45 mL 10% formaldehyde, followed by vortexing for approximately 3 s. This mixture was immersed in a boiling water bath for 15 min and cooled in an ice-water bath for at least 5 min to yield 2,6-diacetyl-1,5-dimethyl-7-（2-carboxyethyl）-3H-pyrrolizine. The reaction mixture was passed through a 0.45-μm microfilter, and an aliquot of filtrate（20 μL）was injected directly into a HPLC system, consisting of a Tosoh TSK-GEL ODS-80Ts column（250 mm × 4.6 mm I.D., particle size 5 μm）maintained at 40° C and eluted with methanol:acetic acid:water（90:4:106, v/v/v）at a flow rate of 0.6 mL/min. The excitation wavelength was 363 nm and the emission wavelength 473 nm. In these conditions, the fluorescent derivative of 5-ALA was eluted after 22.2 min.

Statistical analysis

Pearson coefficients were calculated to determine the correlation between urinary excretion of 5-ALA and energy intake, some nutrient intakes, and urinary excretion amounts of some vitamins. A p-value < 0.05 was considered statistically significant. All analyses were performed using Graph Pad Prism version 5.0（Graph Pad Software, San Diego, CA, USA）.

RESULTS

Relationship between urinary excretion of 5-ALA and energy intake

Urinary 5-ALA excretion per day in the 28 healthy female students（n = 28）ranged from 2.07 μmol/d to 9.86 μmol/d, with a median of 6.17 μmol/d. Figure 2-A shows the relationship between urinary excretion of 5-ALA and energy intake. The average energy intake was 1434 ± 370（mean ± SD）kcal/d. This relationship was not significant.

Relationship between urinary excretion of 5-ALA and major nutrient intakes

The relationships between urinary excretion of 5-ALA and protein intake, fat intake, and carbohydrate intake are shown in Figs. 2-B, 2-C, and

2-D, respectively. These three relationships were not significant. The average intakes of protein, fat, and carbohydrate were 52.4 ± 14.5 g/d, 43.6 ± 20.0 g/d, and 210 ± 73 g/d, respectively.

Relationship between urinary excretion of 5-ALA and some vitamin intakes

Figure 3 shows the relationships between urinary excretion of 5-ALA and some B-group vitamins such as vitamin B_1, vitamin B_2, vitamin B_6, niacin, and vitamin B_{12} which are involved in the biosynthesis of succinyl-CoA, a precursor of 5-ALA (see Fig. 1). These relationships were not significant. However, a trend was observed between urinary excretion of 5-ALA and niacin intake (Fig. 3-D). The average intakes of vitamin B_1, vitamin B_2, vitamin B_6, niacin, and vitamin B_{12} were 0.74 ± 0.26 mg/d, 0.99 ± 0.45 mg/d, 0.96 ± 0.34 mg/d, 21.4 ± 1.84 mg niacin equivalent (NE)/d, and 2.9 ± 1.8 μg/d, respectively.

Relationship between urinary excretion of 5-ALA and urinary excretion amounts of some vitamins

Figure 4 shows urinary amounts of vitamin B_1, vitamin B_2, 4-PIC (a catabolite of vitamin B_6), and vitamin B_{12}. Urinary 5-ALA was not significantly related to urinary excretion of vitamin B_1 (Fig. 4-A), vitamin B_2 (Fig. 4-B) or vitamin B_{12} (Fig. 4-D). However, urinary 5-ALA showed a significant association with urinary excretion of 4-PIC (Fig. 4-C). The average urinary excretion of vitamin B_1, vitamin B_2, 4-PIC, and vitamin B_{12} was 1.19 ± 0.61 μmol/d, 0.55 ± 0.47 μmol/d, 3.99 ± 2.32 μmol/d, and 31.9 ± 1.9 nmol/d, respectively.

Relationship between urinary excretion of 5-ALA and urinary excretion amounts of nicotinamide catabolites

Figure 5 shows the relationship between urine 5-ALA and nicotinamide and its metabolites. As has been reported previously (*16*), urinary excretion of nicotinamide itself was below the limit of detection. A significant correlation was observed between 5-ALA and each of the catabolites of nicotinamide. The strongest relationship was observed between SUM (i.e., MNA + 2-Py + 4-Py) and 5-ALA (Fig. 5-D). The average urinary excretion of MNA, 2-Py, 4-Py, and the SUM (MNA + 2-Py + 4-Py) was 30.7 ± 14.0 μmol/d, 42.5 ± 20.4 μmol/d, 5.6 ± 2.7 μmol/d, and 79.5 ± 39.5μmol /d, respectively.

DISCUSSION

The volunteers in the present experiment were within normal ranges physically and in nutrient intake. They had urinary 5-ALA levels similar to those previously reported (*15*). Reference values of water-soluble vitamins for maintaining health have been reported to be 200–2000 nmol/d each for vitamins B_1 and B_2, 2–15 μmol/d for 4-PIC (a catabolite of vitamin B_6), and 50–300 μmol/d for the sum of nicotinamide catabolites MNA, 2-Py, and 4-Py (*17*). Of the 28 volunteers, three had higher than the reference values for vitamin B_1, and seven, one, and four had lower than the reference values for vitamin B_2, 4-PIC, and the sum of the nicotinamide catabolites, respectively. The elimination route of vitamin B_{12} is not via urine; therefore, we do not have

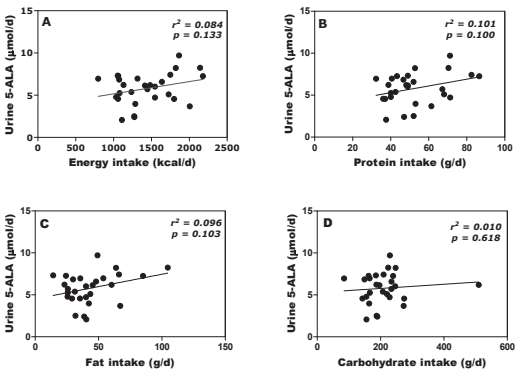

Figure 2. Relationship between urinary excretion of 5-ALA and energy intake (A), protein intake (B), fat intake (C), and carbohydrate intake (D).

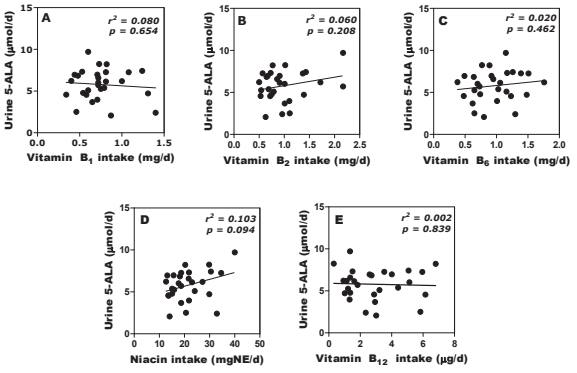

Figure 3. Relationship between urinary excretion of 5-ALA and vitamin B_1 intake (A), vitamin B_2 intake (B), vitamin B_6 intake (C), niacin intake (D), and vitamin B_{12} intake (E).

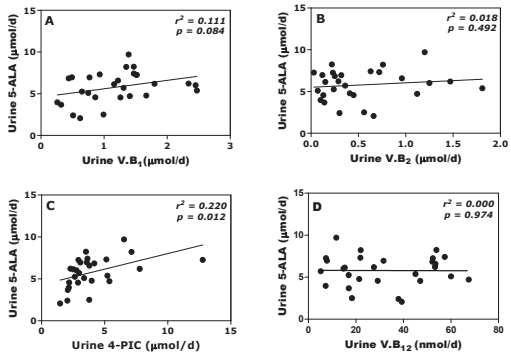

Figure 4. Relationship between urinary excretion of 5-ALA and urinary excretion of vitamin B_1（A）, vitamin B_2（B）, 4-pyridoxic acid［4-PIC, a catabolite of vitamin B_6］（C）, and vitamin B_{12}（D）.

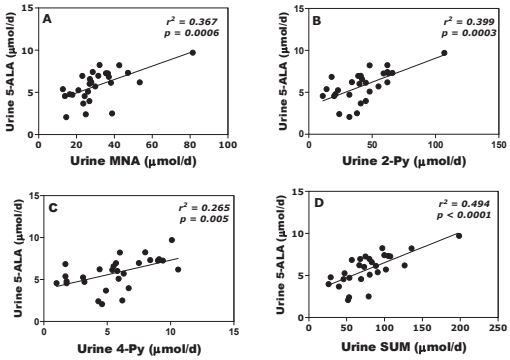

Figure 5. Relationship between urinary excretion of 5-ALA and urinary excretion of N^1-methylnicotinamide［MNA］（A）, N^1-methyl-2-pyridone-5-carboxamide［2-Py］（B）, N^1-methyl-4-pyridone-3-carboxamide［4-Py］（C）, and of the SUM［i.e., MNA + 2-Py + 4-Py］（D）.

a reference value for urinary excretion of vitamin B_{12}（*18*）. From these findings, we judged that the participants in this experiment were representative of young adult women.

We found that urinary 5-ALA levels were strongly correlated with urinary levels of nicotinamide catabolites such as MNA, 2-Py, and 4-Py, and weakly, but significantly, correlated with urinary 4-PIC. Urinary 5-ALA did not correlate with urinary concentrations of vitamins B_1 and B_2. Greater urinary 5-ALA excretion may indicate increased synthesis of 5-ALA, or reduced activity of 5-ALA dehydratase（*18,19*）which needs Zn^{2+}（*20*）.

In toxicology, an increased 5-ALA level suggests lead（Pb）exposure because Pb inhibits 5-ALA dehydratase activity（*21*）. Exposure to Pb also inhibits the activity of NAD^+ synthetase（*22*）which needs Mg^{2+} and K^+（*23*）and is an enzyme involved in the biosynthesis of NAD^+ from tryptophan（*23*）that catalyzes the reaction nicotinic acid adenine dinucleotide + glutamine + ATP → NAD^+ + glutamic acid + ADP + H_3PO_4. The free form of nicotinamide is formed only via NAD^+ in the tryptophan-nicotinamide pathway. If the participants in our study had been exposed to Pb, it is probable that the increased urinary excretion of 5-ALA would be concomitant with decreased urinary excretion of nicotinamide metabolites. However, our data showed increased urinary excretion of both 5-ALA and nicotinamide and, further, a direct relationship was observed between them. Although we did not measure the blood Pb concentration or the relationship between urinary excretion of 5-ALA and Pb intake, we can conclude that the increased urinary excretion of 5-ALA was not due to the inhibition of 5-ALA dehydratase activity by Pb. Therefore, the present data showing increased urinary excretion of 5-ALA indicate a real elevation of 5-ALA synthesis from precursors such as glycine and succinyl-CoA. 5-ALA synthase is a pyridoxal phosphate-dependent enzyme（*2*）. The relationship between the urinary excretion of 4-PIC, a catabolite of vitamin B_6, and the urinary excretion of 5-ALA was significant.

As Fig. 1 shows, some B-group vitamins are involved in the synthesis of succinyl-CoA. Better nutritional status regarding B-group vitamins could increase the synthesis of succinyl-CoA. Urinary excretion of vitamins indicates surplus vitamin intake（*17*）, and higher urinary excretion of vitamins indicates better nutritional status of vitamins. Our data suggest that administration of some nutrients such as niacin and vitamin B_6（as well as some amino acids）may help to synthesize 5-ALA. As mentioned above, 5-ALA plus blue light illumination for the treatment of various premalignant and malignant lesions（*1,2*）, the use of 5-ALA for improvement of glucose intolerance（*3,4*）, and administration of 5-ALA to improve sarcopenia（*5*）have been reported. Therefore, some physiological functions of niacin and vitamin B_6 may actually arise through 5-ALA, a direct precursor of heme. Heme is a cofactor of proteins such as hemoglobin, cytochrome P450s,

other cytochromes（e.g. in the respiratory chain）, catalase, and oxygenases（see Fig. 1）. In particular, two oxygenases（tryptophan 2,3-dioxygenase（*24*）, indoleamine 2,3-dioxygenase（*24*））are involved in the biosynthesis of nicotinamide from tryptophan.

In the present experiment involving humans, a strong, directly proportional relationship was observed between the urinary excretion amounts of 5-ALA and nicotinamide catabolites. This result shows a direct relationship between the syntheses of 5-ALA from succinyl-CoA + glycine and nicotinamide from tryptophan.

Acknowledgments

We thank the volunteers who participated in this study. This investigation was part of the project "Studies on the nutritional evaluations of amino acids and B-group vitamins"（principal investigator, Katsumi Shibata）, which was supported by a Research Grant from Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences［grant number 24300258］. We thank James Allen, DPhil, from Edanz Group（www.edanzediting.com/ac）for editing a draft of this manuscript.

Author contributions

K. S. designed the study. N. M. conducted the experiments. K. S. drafted the manuscript. Both authors read and approved the final manuscript.

Disclosure statement

No potential conflicts of interest were reported by the authors.

REFERENCES

1）Smith S, Piacquadio D, Morhenn V, Atkin D, Fitzpatrick R. 2003. Short incubation PDT versus 5-FU in treating actinic keratoses. *J Drugs Dermatol* 2: 629–635.

2）Yu C-H, Yu C-C. 2014 Photodynamic therapy with 5-aminolevulinic acid（ALA）impairs tumor initiating and chemo-resistance property in head and neck cancer-derived canser stem cells. *PLos ONE* 9: e87129.

3）Rodriguez BL, Curb JD, Davis J, Shintani T, Perez MH, Apau-Ludlum N, Johnson C, Harrigan RC. 2012. Use of the dietary supplement 5-aminiolevulinic acid（5-ALA）and its relationship with glucose levels and hemoglobin A1C among individuals with prediabetes. *Clin Transl Sci* 5: 314–320.

4）Sato T, Yasuzawa T, Uesaka A, Izumi Y, Kamiya A, Tsuchiya K, Kobayashi Y, Kuwahata M, Kido Y. 2014. Type 2 diabetic conditions in Otsuka Long-Evans Tokushima Fatty rats are ameliorated by 5-aminolevulinic acid. *Nutr Res* 34: 544–551.

5）Fujii C, Miyashita K, Mitsuishi M, Sato M, Fujii K, Inoue H, Hagiwara A, Endo S, Uto A, Ryuzaki M, Nakajima M, Tanaka T, Tamaki M, Muraki A, Kawai T, Itoh H. 2017. Treatment of sarcopenia and glucose intolerance through mitochondrial activation by 5-aminolevulinic acid. *Sci Rep* 7:4013. doi: 10.1038/s41598-017-03917-0.

6）Bergonia HA, Franklin MR, Kushner JP, Phillips JD. 2015. A method for determining δ -aminolevulinic acid synthase activity in homogenized cells and tissues. *Clin Biochem* 48: 788–795.

7）Pullman ME, Colowick SP. 1954. Preparation of 2- and 6-pyridones of N^1-methylnicotinamide. *J Biol Chem* 206: 121–127.

8）Shibata K, Kawada T, Iwai K. 1998. Simultaneous micro-determination of nicotinamide and its major metabolites, N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide, by high-performance liquid chromatography. *J Chromatogr* 424: 23–28.

9）Imai T, Sakai S, Mori K, Ando F, Niino N, Shimokata H. 2000. Nutritional assessments of 3-day dietary records in National Institute for Longevity Sciences--Longitudinal Study of Aging（NILS-LSA）. *J Epidemiol* 10: S70–S76.

10）Iwata H, Matsuda T, Tonomura H. 1988. Improved high-performance liquid chromatographic determination of thiamine and its phosphate esters in animal tissues. *J Chromatogr* 26: 317–323.

11）Shibata K, Hirose J, Fukuwatari T. 2014. Relationship between urinary concentrations of nine water-soluble vitamins and their vitamin intakes in Japanese adult males. *Nutr Metab Insights* 7: 61–75.

12）Ohakawa H, Oishi N, Yagi K. 1982. A simple method for micro-determination of flavin in human serum and whole blood by high-performance liquid chromatography. *Biochem Int* 4: 187–194.

13）Gregory JF 3rd, Kirk JR. 1979. Determination of urinary 4-pyridoxic acid using high performance liquid chromatography. *Am J Clin Nutr* 32: 879–883.

14）Shibata K. 1987. Ultramicro-determination of N^1-methylnicotinamide in urine by high-performance liquid chromatography. *Vitamins*（Japanese）61: 599–604.

15）Oishi H, Nomiyama H, Nomiyama K, Tomokuni K. 1996. Fluorometric HPLC determination of delta-aminolevulinic acid（ALA）in the plasma and urine of lead workers: biological indicators of lead exposure. *J Anal Toxicol* 20: 106–110.

16）Shibata K, Onodera M, Shimada S, Yasuda K. 1992. Changes in nicotinamide metabolism and its bioavailability during long-term administration of a multivitamin preparation. *Vitamins（Japan）* 66: 309–314.

17）Shibata K, Fukuwatari T. 2013. Values for evaluating the nutritional status of water-soluble vitamins in humans. *J Integrates OMICS* 3: 60–69.

18）Goering PL, Mistry P, Fowler BA.1986. A low molecular weight lead-binding protein in brain attenuates lead inhibition of delta-aminolevulinic acid dehydratase: comparison with a renal lead-binding protein. *J Pharmacol Exp Ther* 237: 220–225.

19）Sasaki T. 2000. Biomarkers of lead exposure. *Industrial Health* 38: 127–142.

20）Jaffe EK, Salowe SP, Chen NT, DeHaven PA. 1984. Porphobilinogen synthetase modification with methylmethanethiosulfonate. A protocol for the investigation of metalloproteins. *J Biol Chem* 259: 5032–5036.

21）Sakai T, Yanagihara S, Ushio K. 1981. Erythrocyte factors concerned in th einhibition of ALA-D by lead. *Br J Ind Med* 38: 268–274.

22）Zerez CR, Wong MD, Tanaka RR. 1990. Partial purification and properties of nicotinamide adenine dinucleotide synthetase from human erythrocytes: evidence that enzyme activity is a sensitive indicator of lead exposure. *Blood* 75: 1576–1582.

23）Yu CK, Dietrich LS. 1972. Purification and properties of yeast nicotinamide adenine dinucleotide synthetase. *J Biol Chem* 247: 4794–4802.

24）Yan D, Lin YW, Tan X. 2017. Heme-containing enzymes and inhibitors for tryptophan metabolism. Metallomics 9：1230–1240.